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14. ABSTRACT Obesity in postmenopausal women is associated with increased breast cancer risk, development of more aggressive tumors and resistance to certain anti-breast cancer treatments. These effects might be mediated by obesity hormone leptin. Here we investigated if leptin can transactivate the oncogenic receptor HER2 and interfere with the activity of anti-HER2 antibody. We found that HER2 and the leptin receptor (ObR) are coexpressed in several studied breast cancer cell lines. In MCF-7 cells, HER2 physically interacted with ObR and leptin treatment increased HER2 phosphorylation on Tyr 1248. Furthermore, leptin reduced the efficacy of anti-HER2 drug Herceptin. Studies of human breast cancers revealed that the presence of leptin correlated with ObR, and the whole leptin system was coexpressed with HER2 in ~50% of all tumors. Thus, coexpression of HER2 and the leptin/ObR system might contribute to enhanced HER2 activity and reduced sensitivity to anti-HER2 treatments.					
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Introduction

Obesity has been shown to be associated with increased breast cancer risk in postmenopausal women, development of more aggressive breast tumors and resistance to certain anti-breast cancer treatments [1]. The molecular mechanisms of these effects are still not clear. Recent data suggested that excess body weight could promote breast cancer through increased production of an adipocyte-derived hormone leptin [2]. The purpose of this work was to assess whether leptin can transactivate the oncogenic receptor HER2 in breast cancer cells, and if leptin/HER2 interaction could occur in vivo, in human breast cancer. Consequently, the objectives of this proposal were:

- 1) To examine the expression of leptin receptors (ObRl and ObRs) in anonymized HER2-positive breast tumor biopsies;
- 2) To establish correlations between each leptin, ObRl and ObRs and tumor characteristics;
- 3) To examine in breast cancer cell models if activation of the leptin receptor can induce HER2 phosphorylation and downstream signaling in the presence and absence of Herceptin;
- 4) To determine if leptin transactivation of HER2 might be blocked with ObR antagonists.

Body

The work progressed along the tasks listed in SOW. The performance period of this award has been extended till August 31, 2009, in part to evaluate the expression of the leptin system in triple-negative breast cancer.

The results that we obtained to date are:

1) We found that ObR and HER2 are coexpressed in breast cancer cell lines. We examined whether similar interactions could occur in breast cancer cell models. To this end, we tested ObRl and ObRs expression in four different cell lines with varying levels of HER2: BT-474 and SK-BR-3 cells, known to express high levels of HER2, and MCF-7 and ZR-75 cell lines characterized by moderate HER2 expression (Fig. 1, Fiorio et al., manuscript attached). The expression of the signaling ObRl isoform (~190 kDa) as well as two short ObR isoforms (~150 and 160 kDa) was confirmed in MCF-7 cells. Low levels of ObRl were also found in BT-474 cells, while minimal expression of ObRl was detected in SK-BR-3 and ZR-75 cells. All cell lines expressed different isoforms of ObRs (Fig. 1, Fiorio et al.).

2) We found that leptin treatment transactivates HER2. To study whether leptin can transactivate HER2, we focused on

MCF-7 cell line as it expresses both HER2 and high levels of ObR1 and ObRs. The acute stimulation of MCF-7 cells with leptin induced HER2 phosphorylation on Tyr1248. Leptin-dependent activation of Tyr1248-HER2 was the strongest with 200 ng/mL leptin, but HER2 was found phosphorylated also with 100 and 500 ng/mL leptin (Fig. 2, Fiorio et al.). The highest doses of leptin (500 ng/mL) induced rapid downregulation of HER2, most likely due to ligand-dependent internalization [3]. Similar induction of Tyr1248-HER2 was observed with EGF, a known activator of this receptor [4] (Fig. 2, Fiorio et al.). Like with leptin, the best stimulation of HER2 was seen with the 200 ng/mL dose and high EGF concentrations produced HER2 downregulation.

3) We determined that ObR and HER2 colocalize and coprecipitate in breast cancer cells. Using specific immunofluorescence staining combined with confocal microscopy, we found that HER2 colocalizes with ObR in MCF-7 cells (Fig. 3A, Fiorio et al.). The colocalization was detected in $20 \pm 0.7\%$ of cells. In addition, we found that HER2 can be detected in ObR immunoprecipitates obtained from growing MCF-7 cells (Fig. 3B, Fiorio et al.).

4) We found that treatment of MCF-7 cells with anti-HER2 Ab (Herceptin) decreases the levels of HER2 through the process of ubiquitination and proteasomal degradation, while addition of leptin interferes with the effect of Herceptin (Fig. I A,B).

5) We evaluated breast cancer biopsies and found that the leptin/ObR system is coexpressed with HER2 in a large subgroup of breast cancers (50%). Specifically, we analyzed the expression of leptin and ObR by IHC in HER2-positive and HER2-negative breast cancers. This screening demonstrated that both leptin and ObR can be expressed in both HER2-positive and HER-2-negative tumors (Tab. 2, Fig. 4, Fiorio et al.). Statistical analysis did not reveal any significant associations between leptin or ObR and HER2. However, correlations between the leptin/ObR system and HER2 and other markers should be re-evaluated in the future in a larger group of biopsies.

6) Because we detected the leptin system in HER2-negative tumors, we evaluated by immunohistochemistry the expression of leptin and ObR in a larger collection of triple-negative (HER2-, estrogen receptor-, progesterone receptor-negative) breast cancers, the cancers of a particularly aggressive phenotype for which no targeted

therapy exist. Analysis of 78 cases of triple-negative tumors revealed that leptin and ObR were expressed in 34% of cases. In addition leptin and ObR were coexpressed in 89% of those cases. The evaluation of other markers that are associated with the leptin system in triple-negative tumors is underway.

The materials and methodology used in the above studies were described in detail in the attached manuscript (Fiorio et al.) and in ref. [5].

Key Research Accomplishments

- 1) Established that HER2 and the leptin/ObR system are coexpressed in ~50% of breast cancers;
- 2) Demonstrated that HER2 and ObR are coexpressed in breast cancer cell lines;
- 3) Demonstrated that HER2 and ObR can physically interact in breast cancer cells;
- 4) Found that high concentrations of leptin can transactivate HER2 in breast cancer cells;
- 5) Found that leptin treatment reduces the efficacy of trastuzumab (Herceptin) in vitro;
- 6) Found that leptin reduces ubiquitination and increases HER2 stability in the presence or absence of trastuzumab;
- 7) Found that the leptin system is expressed in ~34% of triple-negative breast tumors.

Reportable Outcomes

- 1) Manuscript **"LEPTIN/HER2 CROSSTALK IN BREAST CANCER: IN VITRO STUDY AND PRELIMINARY IN VIVO ANALYSIS"** by Elena Fiorio, Anna Mercanti, Marianna Terrasi, Rocco Micciolo, Andrea Remo, Alessandra Auriemma, Annamaria Molino, Veronica Parolin, Bruno Di Stefano, Maria Franco Bonetti, Antonio Giordano, Gian Luigi Cetto, **Eva Surmacz**, BMC Cancer,
- 2) Abstract **"FUNCTIONAL RELATIONSHIPS BETWEEN HER2 AND THE LEPTIN (OBESITY) SYSTEM IN BREAST CANCER"**, presented by **Eva Surmacz** at DOD "Era of Hope" Meeting, Baltimore, June 26, 2008;
- 3) Invited talk **"Leptin and breast cancer"** by **Eva Surmacz**, University of Palermo, Italy, May 13, 2008.
- 4) Invited talk **"Leptin and cancer"** by **Eva Surmacz**, Princess Takamatsu Cancer Research Symposium, Tokyo, November 11-15, 2008.
- 5) Invited talk **"Leptin interference with cancer therapeutics"**, University of Palermo, Italy, May 10, 2009.

Conclusion

High levels of obesity hormone leptin can activate HER2 and possibly interfere with anti-HER2 therapies. Our further studies will evaluate if anti-leptin strategies could become beneficial in the treatment of Herceptin (trastuzumab)-resistant breast cancer, especially in obese patients. In addition, because the leptin system is expressed in a fraction of triple-negative breast cancer, targeting ObR could provide a novel option for targeted therapy in those cases.

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Appendices

- 1) Fig I A,B. Leptin interference with trastuzumab (Herceptin) treatment in vitro.
- 2) Manuscript "LEPTIN/HER2 CROSSTALK IN BREAST CANCER: IN VITRO STUDY AND PRELIMINARY IN VIVO ANALYSIS".

Supporting Data

N/A

Fig. I. Effects of leptin on trastuzumab-mediated HER2 degradation. A. MCF-7 cells were treated with 200 ng/mL leptin or 15 ug/mL trastuzumab (Herceptin) for 0-72 h. The expression of HER2 and the levels of control protein GAPDH were detected by WB, as described in Fiorio et al. 2008 (manuscript attached). B. The cells were pre-treated with proteasomal inhibitor MG-132 (10 μ M) for 24 h and then treated with trastuzumab in the presence or absence of leptin for 1 h. Upon treatment, HER2 was immunoprecipitated from 500 μ g proteins and its poly-ubiquitination levels (Ubq) were analyzed using anti-Ubq antibodies. The technology for protein ubiquitination analysis was described in detail by us in ref. [5].

Fig. IA

Leptin Stabilizes, Trastuzumab Downregulates, HER2 in MCF-7 Cells

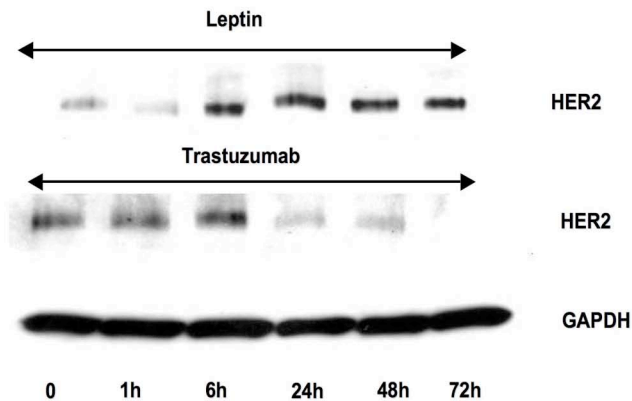
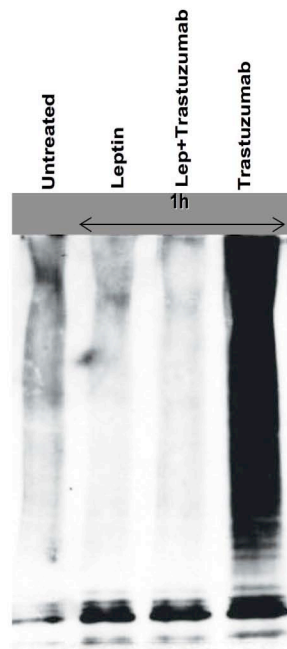


Fig. 1B

Leptin Blocks Trastuzumab-induced HER2 Downregulation

Leptin decreases ubiquitination of HER2 in the absence or presence of trastuzumab



Research article

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Leptin/HER2 crosstalk in breast cancer: *in vitro* study and preliminary *in vivo* analysis

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Abstract

Background: Obesity in postmenopausal women is associated with increased breast cancer risk, development of more aggressive tumors and resistance to certain anti-breast cancer treatments. Some of these effects might be mediated by obesity hormone leptin, acting independently or modulating other signaling pathways. Here we focused on the link between leptin and HER2. We tested if HER2 and the leptin receptor (ObR) can be coexpressed in breast cancer cell models, whether these two receptors can physically interact, and whether leptin can transactivate HER2. Next, we studied if leptin/ObR can coexist with HER2 in breast cancer tissues, and if presence of these two systems correlates with specific clinicopathological features.

Methods: Expression of ObR, HER2, phospho-HER2 was assessed by immunoblotting. Physical interactions between ObR and HER2 were probed by immunoprecipitation and fluorescent immunostaining. Expression of leptin and ObR in breast cancer tissues was detected by immunohistochemistry (IHC). Associations among markers studied by IHC were evaluated using Fisher's exact test for count data.

Results: HER2 and ObR were coexpressed in all studied breast cancer cell lines. In MCF-7 cells, HER2 physically interacted with ObR and leptin treatment increased HER2 phosphorylation on Tyr 1248. In 59 breast cancers, the presence of leptin was correlated with ObR (the overall association was about 93%). This result was confirmed both in HER2-positive and in HER2-negative subgroups. The expression of leptin or ObR was numerically more frequent in larger (> 10 mm) tumors.

Conclusion: Coexpression of HER2 and the leptin/ObR system might contribute to enhanced HER2 activity and reduced sensitivity to anti-HER2 treatments.

Background

Recent epidemiological and clinical data confirmed that obesity in postmenopausal women is associated with increased breast cancer risk, development of more aggressive breast tumors and resistance to certain anti-breast cancer treatments [1-4]. The molecular mechanisms of this link are not clear, but several studies in animal and cellular models suggested that excess body weight could promote breast cancer through increased production of an adipocyte-derived hormone leptin [5-7]. The primary function of leptin is to regulate energy balance and food intake by acting in the brain, but the hormone also plays an important role in peripheral organs, modulating fertility, lactation, and immune response [8,9]. Leptin levels in humans correlate with adiposity and are usually higher in females than in males [8].

Leptin action is mediated through the transmembrane leptin receptor ObR [10]. The human ObR can be expressed as at least four isoforms with different COOH-terminal cytoplasmic domains [11]. The full (long) form of ObR (ObRl) contains the extracellular, transmembrane, and intracellular domains [10]. Only ObRl has a full signaling potential, while the short ObR isoforms (ObRs) have diminished or abolished signaling activity [12]. ObRl is highly expressed in the hypothalamus, however lower levels of ObRl have been identified in many peripheral tissues [5,13-15]. The major pathways activated by ObRl are the classic cytokine JAK2/STAT3 pathway, the Ras/ERK1/2 signaling cascade, and the PI-3K/Akt/GSK3 growth/anti-apoptotic pathway [12].

Recently, leptin has been found to be involved in neoplastic processes, especially in breast carcinogenesis [5-7,16]. Specifically, leptin can promote cancer cell growth and transformation in vitro and in vivo, and increase cell survival in the presence of anti-cancer drugs [5,17]. The role of leptin in breast cancer has been substantiated by the fact breast tumors, but not normal mammary epithelium, overexpress both leptin and ObR [18-20], and the leptin/ObR system correlates with higher tumor grade and worse prognosis [18,19]. In addition, intratumoral levels of ObRl and ObRs and high levels of serum leptin were found to correlate with poor prognosis [21].

Leptin may exert its activity not only through ObR, but also through crosstalks with other signaling systems. For instance, leptin affects the synthesis and/or function of estrogen receptor alpha (ER α), vascular endothelial growth factor (VEGF), and human epidermal growth factor receptor 2 (HER2) [5,6,22-25]. Leptin may also promote tumor cell survival in xenograft models via increased expression of E-cadherin [17].

HER2 is a tyrosine kinase that is amplified in 25–30% of breast tumors and its overexpression often correlates with a more aggressive, metastatic phenotype and worse prognosis [26,27]. Current therapies of HER2-positive tumors include treatments with trastuzumab (Herceptin), a monoclonal HER2 antibody, but resistance to this drug is a common problem that ultimately leads to treatment failure [28]. The molecular basis of trastuzumab resistance are still obscure, but there is evidence that increased activation of other growth factor signaling systems may contribute to this process [28].

Preliminary data obtained in human embryonic kidney HEK 293T cells engineered to coexpress HER2 and ObRs or ObRl suggested that leptin, acting through either ObR isoform, can rapidly induce HER2 phosphorylation and activation of HER2 intracellular signaling [22]. Recent data of Soma et al. suggested that in SK-BR-3 breast cancer cells, leptin can transactivate HER2 through both the epidermal growth factor receptor HER1 and JAK2 pathways [29]. Thus, transactivation of HER2 by liganded ObR or by HER1 might constitute an important mechanism of HER2 resistance in breast cancer patients, especially those expressing high levels of leptin and ObR in breast cancer tissues. However, the existence of functional leptin/HER2 interactions in human breast cancer has not been explored.

Consequently, using breast cancer cell lines naturally expressing HER2 and ObR, we tested if HER2 and ObR can physically interact and if exposure of cells to leptin can transactivate HER2. To validate in vitro data, we studied whether the leptin/ObR system and HER2 can be coexpressed in breast cancer biopsies and if coexpression of these two systems is associated with specific clinicopathological features.

Methods

Patients and tissue samples

The expression of leptin, ObR, and other markers was assessed in breast cancer samples. Tissue samples were obtained from 59 women (31 HER2-positive and 28 HER2-negative) who underwent partial or total mastectomy and lymph node dissection for primary breast cancer at the University and Public Hospitals in Verona between January 1, 1992 and November 15, 2006 (Table 1). All of the patients had a histologically confirmed diagnosis of breast cancer. Patients with a histological diagnosis of breast sarcomas and males with breast cancer were excluded from the analysis. Clinical staging was applied according to the sixth edition of the Union International Contre le Cancer/American Joint Committee on Cancer TNM classification manual [30]. All tissue samples were anonymized and the local ethical committee approved the study protocol.

Table 1: Patient and tumor characteristics

Clinicopathological Features	Patients n (%)	Clinicopathological Features	Patients n (%)
Menopausal status		T	
Postmenopausal	15 (25)	TIS	3 (5)
Premenopausal	39 (66)	pTx	1 (2)
Unknown	5 (9)	pT1	19 (33)
Histotype		pT2	22 (37)
Ductal invasive	45 (76)	pT3	2 (3)
Lobular invasive	4 (7)	pT4	12 (20)
Intraductal	3 (5)	Diameter (mm)	
Inflammatory	4 (7)	≤ 10 mm	9 (15)
Other	3 (5)	> 10 mm	42 (71)
Grading		Unknown	8 (14)
G1	7 (12)	N	
G2	18 (31)	pN1-3	20 (34)
G3	26 (44)	pN4-10	9 (15)
Unknown	8 (13)	pN > 10	4 (7)
Ki-67		Negative	19 (32)
0-15%	33 (56)	Unknown	7 (12)
16-25%	9 (15)	HER-2	
26-100%	14 (24)	Positive	31 (53)
Unknown	3 (5)	Negative	28 (47)
ER/PgR		LVI	
ER+/PgR+	38 (64)	Positive	27 (46)
ER-/PgR-	12 (20)	Negative	24 (41)
ER+/PgR-	7 (12)	Unknown	8 (13)
ER-/PgR+	1 (2)		
Unknown	1 (2)		

G, differentiation grade; G1-3, low, moderate or intermediate, high grade. ER, estrogen receptor alpha; PgR, progesterone receptor; T, tumor size; pT1, 0-2 cm; pT2, 2-5 cm; pT3, > 5 cm; pT4, ulcerated or attached; Tis, carcinoma in situ; pTx, primary tumor of unknown pT; N, node status; LVI, lymphovascular invasion; n, number of cases.

Pathological features

Diameter of the tumors was measured in millimeters, their grading (G) was classified as standard G1, G2, G3; node involvement was defined as positive ($N \geq 1$) (cancer cells found in one or more lymph nodes) or negative ($N = 0$) (absence of regional metastases). We further evaluated lymphovascular invasion (LVI), classified as positive or negative according to the presence or absence of tumor cells in the lumens of lymphatic or blood vessels (Table. 1).

Biological features

In all cases, serial-step 5 μ m sections were cut from paraffin-embedded tissue samples and stained with hematoxylin-eosin for histological examination. ER α and progesterone receptor (PgR) status was determined by immunohistochemistry (IHC). Tumors expressing at least 1% of cells positive for ER α or PgR were considered positive, according to recommendations of 10th St Gallen Conference on Primary Therapy of Early Breast Cancer [31]. IHC staining for the replicative cell fraction was performed using a Ki-67 monoclonal antibody (mAb) (DAKO, Denmark). Ki-67 expression results were arbitrarily classified as low ($\leq 15\%$ of stained cells), medium (16-25%) or high ($> 26\%$). HER2 levels were determined by

IHC using the HercepTest (DAKO). HER2 expression levels obtained by IHC were scored as 0 (no staining), 1+ (faint incomplete membranous pattern), 2+ (moderate complete membranous pattern) and 3+ (strong membranous pattern). Samples with scores 0 and 1+ were considered HER2-negative and with the score 3+ were considered HER2-positive. To confirm or exclude HER2 positivity, samples with a score 2+ were further evaluated with fluorescence in situ hybridization (FISH) using Path-Vysion assay (Abbott Diagnostics, Rome, Italy). The status of p53 has not been assessed. The characteristics of patients and tumors are summarized in Table. 1.

Detection of leptin and ObR in breast cancer biopsies

The expression of leptin and ObR was investigated by IHC with specific Abs, as described by us before [18]. Briefly, tissue sections were deparaffinized using a dry oven at 60°C overnight, then the slides were dewaxed in xylene and rehydrated in graded series of ethanol. After rinsing in PBS, endogenous peroxidase activity was inhibited by incubation with 30% hydrogen peroxide, diluted in 100% methanol for 30 min at 4°C. After three washes in PBS the sections were incubated with 1.5% blocking serum for 1 h, then the sections were incubated for 3 h with primary antibodies. For leptin staining, we used the A20 leptin

polyclonal Ab (pAb) (Santa Cruz Biotechnology, Santa Cruz, USA) at 1:100 dilution; for ObR staining, the M18 ObR pAb (Santa Cruz Biotechnology) at 1:50 dilution overnight at 4°C. The leptin and ObR antigens were detected with avidin-biotin-peroxidase ABC staining systems (Santa Cruz Biotechnology). All slides were counterstained with Mayer's hematoxylin. Breast specimens previously classified as positive for the expression of the studied markers were used for control and protocol standardization. In negative controls, primary Abs were omitted. Assessment of immunoreactivity was performed in at least 10 different section fields by two independent evaluators by light microscopy, and the mean percentage of tumor cells displaying positive staining was scored. The expression of leptin and ObR in cancer samples was classified using a four-point scale: 0, < 10% stained cells; 1+, 10–50% cells with weak staining; 2+, >50% cells with weak staining; 3+, > 50% cells with strong staining. Tumors with the score of at least 1+ were considered positive for the expression of leptin or ObR.

Cell culture and treatments

Breast cancer cell lines used in this study included MCF-7, SK-BR-3, BT-474 and ZR-75-1 cells, all purchased from the American Type Culture Collection (Rockville, MD, USA). MCF-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM:F12) (Cellgro, Herndon, VA, USA) containing 5% calf serum (CS) and 1% Penicillin/Streptomycin (P/S) (Cellgro). SK-BR-3 cells were grown in DMEM F12 containing 10% fetal bovine serum (FBS) and 1% P/S. BT-474 cells were grown in DMEM:F12 containing 10% FBS, 1% P/S and 0.01 µg/mL insulin. ZR-75-1 cells were grown in RPMI-1640 (BioWhittaker, Walkersville, MD, USA) containing 10% FBS, 1% P/S and 1 mM sodium pyruvate (Cellgro).

For growth factor stimulation, the cells (4.5 to 6.0×10^5 cells/100 mm dish) were placed in phenol red-free serum-free medium (SFM) [32] for 24 h and then stimulated with different doses of leptin (Ob) (R&D systems, Minneapolis, MN, USA) or epidermal growth factor (EGF) (BD Biosciences, Bedford, MA, USA) for 15 min.

Western blotting (WB) and immunoprecipitation (IP)

Total and phospho-HER2 levels were detected by Western Blotting (WB) with Neu C-18 and p-Neu Tyr1248-R Abs (Santa Cruz Biotechnology), respectively. Total protein lysates were obtained as described before [32]. For WB or IP of ObR, we used H-300 Ab (Santa Cruz Biotechnology) recognizing a common domain within ObRl and ObRs and suitable for detection of all ObR isoforms. For WB, we routinely used 35–100 µg of total protein, while for IP, 500 µg of proteins were precipitated using protein A agarose beads (Sigma-Aldrich, St. Louis, MO, USA). IP samples were precleared with protein A agarose for 4 h before

addition of primary Abs. Unrelated IgG was used as negative control for ObR Abs. Otherwise, all WB and IP procedures and measurements of protein abundance followed protocols described in detail before [32]. All WB and IP/WB experiments were repeated at least 3 times.

HER2 and ObR detection by immunofluorescence/deconvoluted microscopy

MCF-7 cells were plated in 2-well Permanox chamber slide (Nunc, Rockester, NY, USA) at a concentration 9×10^5 cells/well. Next day, the cultures were shifted in SFM for 24 h and then treated with leptin 100 ng/mL for 15 min. Then the cells were washed three times with PBS, fixed in 4% paraformaldehyde for 20 min at 4°C, washed again and blocked with 7.5% BSA for 2 h at room temperature. The expression of HER2 was detected using HER2 Neu C-18 Ab 1:100 (Santa Cruz) and donkey anti-rabbit IgG-TRITC 1: 1000 (Santa Cruz); ObR was detected using Ob-R F-18 Ab 1:50 (Santa Cruz) and donkey anti-goat IgG FITC 1:1000 (Santa Cruz). The slides were covered with Vectashield mounting medium containing DAPI (Vector laboratories, IncBurligame, CA, USA) to allow visualization of cells nuclei. The coexpression of HER2 and ObR was assessed using Olympus IX81 deconvoluted microscope and Slidebook software.

Elisa for phospho-HER2

MCF-7 cells were stimulated with 50, 100, 200, 500 ng/mL leptin for 15 min, or were left untreated in SFM. Total proteins were isolated as described for WB. Tyrosine phosphorylated HER2 (Tyr 1248) was measured using DuoSet IC Human Phospho-ErbB2 Elisa kit (R&D Systems, Minneapolis, MN, USA), following manufacturers instructions. 350 µg of sample proteins and 150 ng of the provided Tyr 1248 HER2 protein control were used for measurements. The reading was done using Microplate Autoreader Bio-Tek EL311.

Statistical analysis of data

Relationships among markers studied by IHC were evaluated using Fisher's exact test for count data with a significance level of 0.05 [33]. Statistical analyses were performed using R for Windows software (R Development Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, 2007. ISBN 3-900051-07-0, URL <http://www.R-project.org>). All Elisa and WB assays were done data in triplicate and the results were evaluated using one-way analysis of variance.

Results

ObR and HER2 are coexpressed in breast cancer cell lines

The results obtained in HEK 293T kidney cells engineered to overexpress ObR and HER2 suggested that leptin can transactivate HER2 [22]. Thus, we examined whether sim-

ilar interactions could occur in breast cancer cell models. To this end, we tested ObRl and ObRs expression in four different cell lines with varying levels of HER2: BT-474 and SK-BR-3 cells, known to express high levels of HER2, and MCF-7 and ZR-75 cell lines characterized by moderate HER2 expression (Figure. 1). The expression of the signaling ObRl isoform (~190 kDa) as well as two short ObR isoforms (~150 and 160 kDa) was confirmed in MCF-7 cells. Low levels of ObRl were also found in BT-474 cells, while minimal expression of ObRl was detected in SK-BR-3 and ZR-75 cells. All cell lines expressed different isoforms of ObRs (Figure. 2).

Leptin treatment transactivates HER2

To study whether leptin can transactivate HER2, we focused on MCF-7 cell line as it expresses both HER2 and high levels of ObRl and ObRs ([32] and Figure. 1). As demonstrated by us and others before, MCF-7 cells respond to 100–500 ng/mL leptin with the activation of

different intracellular pathways leading to cell proliferation and survival [32,34].

The acute stimulation (15 min) of MCF-7 cells with leptin induced HER2 phosphorylation on Tyr1248. Leptin-dependent activation of Tyr1248-HER2 was the strongest with 200 ng/mL leptin, but HER2 was found phosphorylated also with 100 and 500 ng/mL leptin (Figure. 2). The highest doses of leptin (500 ng/mL) induced rapid downregulation of HER2, most likely due to ligand-dependent internalization [35]. Similar induction of Tyr1248-HER2 was observed with EGF, a known activator of this receptor [36] (Figure. 2). Like with leptin, the best stimulation of HER2 was seen with the 200 ng/mL dose and high EGF concentrations produced HER2 downregulation. Lower doses of leptin or EGF (10, 25 and 50 ng/mL) did not induce any HER2 response in MCF-7 cell model (data not shown).

Activation of HER2 by leptin was also assessed independently using a specific phospho-HER2 Elisa kit. With this methodology, we found that leptin at 100, 200, and 500 ng/mL induced HER2 phosphorylation on Tyr1248 by 135, 230, and 85%, respectively.

ObR and HER2 colocalize and coprecipitate in breast cancer cells

Next, we probed if HER2 and ObR can physically interact in breast cancer cells. Using specific immunofluorescence staining combined with confocal microscopy, we found that HER2 colocalizes with ObR in MCF-7 cells (Figure. 3A). The colocalization was detected in $20 \pm 0.7\%$ of cells. In addition, we found that HER2 can be detected in ObR immunoprecipitates obtained from growing MCF-7 cells (Figure. 3B).

The leptin/ObR system is coexpressed with HER2 in a large subgroup of breast cancers

The data obtained in breast cancer cell models prompted us to investigate whether ObR isoforms together with their ligand, leptin, can be coexpressed with HER2 in human breast cancer. We analyzed the expression of leptin and ObR by IHC in HER2-positive and HER2-negative breast cancers characterized in Table. 1. This screening demonstrated that both leptin and ObR can be expressed in both HER2-positive and HER2-negative tumors (Table. 2, Figure. 4).

As noted in previous studies from our and other laboratories, leptin and ObR tend to be coexpressed in different cancers [18,19,37,38]. Similarly, the present analysis confirmed strong and significant associations between leptin and ObR in all tumors (Table. 2 and Figure. 4); there were only 4 cancers with a discordant result so that the overall agreement was higher than 93%. The coexpression of lep-

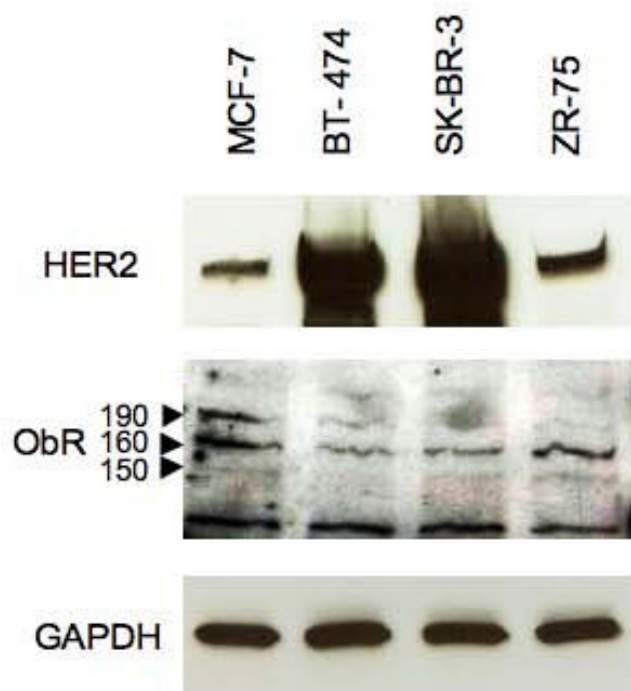
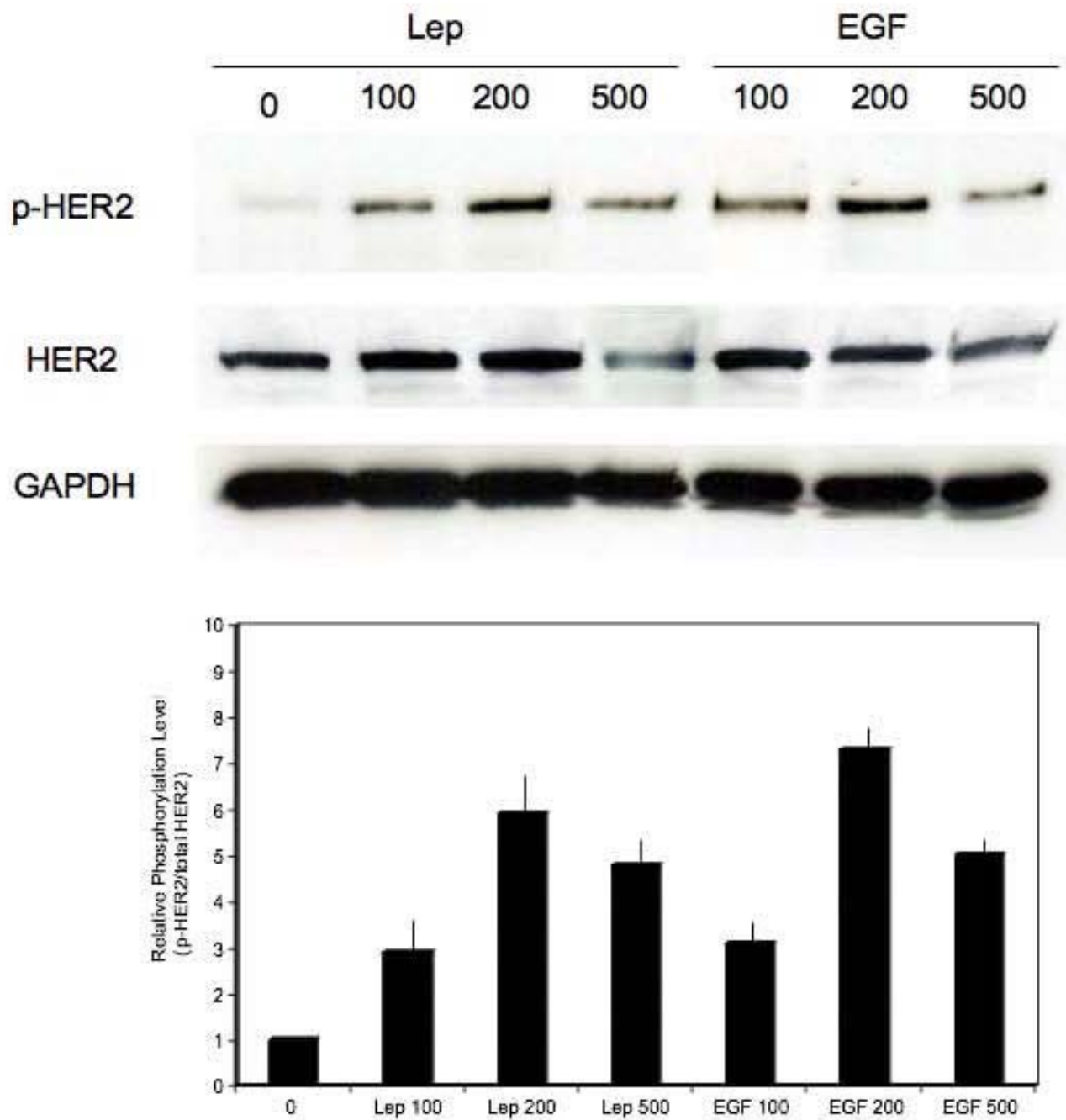
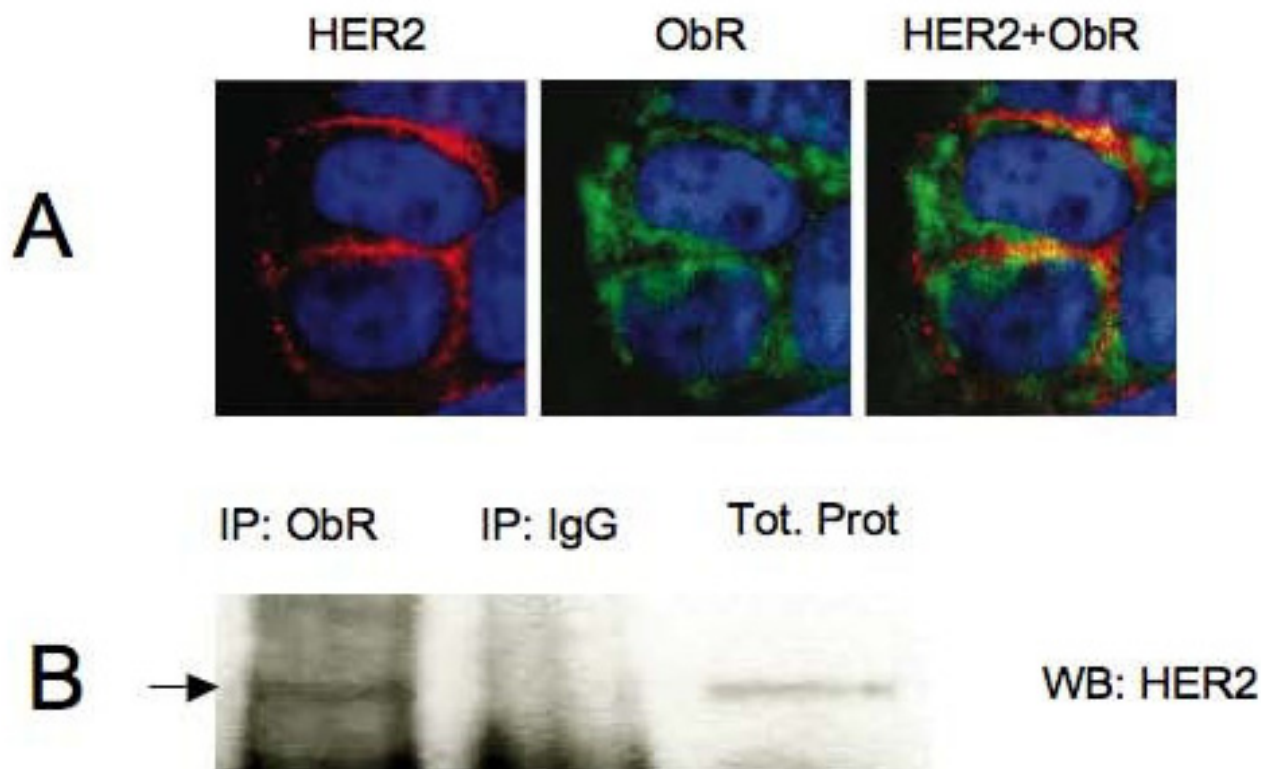


Figure 1
Expression of HER2 and ObR in breast cancer cell lines. The expression of HER2 (185 kDa) and different isoforms of ObR (150–190 kDa, indicated by arrows) was detected in 50 µg of total protein lysates obtained from MCF-7, BT-474, SK-BR-3, and ZR-75 cell lines by WB with specific Abs, as described in Materials and Methods. The presence of a constitutively expressed enzyme GAPDH was assessed in the same blot as control of protein loading.

**Figure 2**

Transactivation of HER2 by leptin. MCF-7 cells were stimulated for 15 min with 100, 200, 500 ng/mL doses of leptin (Lep) or EGF. The expression of Tyr 1248 HER2 (p-HER2), total HER2 levels (HER2) was studied in 100 µg of total proteins by WB with specific Abs, as described in Materials and Methods. The levels of GAPDH in the same blots were assessed to control protein loading. The graph represents levels of Tyr 1248 HER2 relative to total HER2 under different stimuli. Bars represent SE.

**Figure 3**

Physical interactions between ObR and HER2. (A) Growing subconfluent cultures of MCF-7 cells were processed for HER2 (red staining) and ObR (green staining) immunofluorescence as described in Materials and Methods. Colocalization of HER2 and ObR was detected by merging (HER2+ObR) images (yellow staining). Cell nuclei were detected by DAPI (blue staining). (B) Total proteins from growing MCF-7 cells were immunoprecipitated with ObR Abs or control unrelated IgG, as described in Materials and Methods. The presence of HER2 in ObR and IgG IPs was detected by WB and is indicated by arrow. 35 µg of total MCF-7 cell proteins were run on the same gel to control HER2 Ab specificity.

tin and ObR was confirmed both in HER2-positive and in HER2-negative subgroups; the overall agreement was 97% and 89% respectively (Table. 2).

The expression of leptin or ObR was, at least numerically, associated with tumor size, being more frequent in large (> 10 mm) tumors (Table. 3); however, owing to the limited sample size, this relationship was only marginally significant for leptin ($p = 0.061$) and even less significant for ObR ($p = 0.137$). The expression of leptin or ObR was not associated with other considered variables (Table. 3).

Discussion

Obesity increases the risk of postmenopausal breast cancer by 30–50% [2]. Furthermore, excess body weight is associated with poorer survival and increased recurrence of cancer, regardless of menopausal status, after adjustment for stage and treatment [2]. Because of the increas-

ing number of obese breast cancer patients, the mechanism of this phenomenon is currently under thorough investigation. Multiple studies implicated different biologically active substances produced by adipose tissue as possible contributing factors [4,5,7]. Steroid hormones, e.g., estrogens, or growth factors, e.g., insulin-like growth factor I, all of which are secreted by fat cells, are known to promote breast cancer cell growth, transformation and survival [39,40]. New evidence obtained in cellular and animal breast cancer models suggests that leptin, the major hormone produced by the fat tissue, can be mechanistically involved in these neoplastic processes [6].

Although, in view of some inconsistent reports [5,6,21,41,42], the impact of circulating leptin needs further evaluation, one recent study correlated coexistence of high systemic leptin concentrations and high intratumoral ObR mRNA levels with poor prognosis in breast

Table 2: Associations between leptin and ObR in HER2-positive and HER2-negative breast cancer

		ObR +		ObR -		p-value
		N	%	N	%	
All tumors	Leptin +	46	78.0	1	1.7	< 0.001
	Leptin -	3	5.1	9	15.3	
HER2 positive	Leptin +	23	74.2	0	0.0	< 0.001
	Leptin -	1	3.2	7	22.6	
HER2 negative	Leptin +	23	82.1	1	3.6	0.045
	Leptin -	2	7.1	2	7.1	

The expression of leptin and ObR was assessed by IHC, as described in Materials and Methods. The percentage (%) and actual number (N) of tumors expressing combinations of leptin and ObR is given for all tumors as well as in HER2-positive and -negative subgroups. Associations between leptin and ObR in were evaluated using Fisher's exact test for count data.

cancer patients [21]. In addition, breast cancer cells themselves can synthesize leptin in response to obesity-related stimuli [18,43,44]. It remains to be evaluated if the frequent coexpression of leptin and ObR in breast tumors [18-21,45] indeed reflects patient's adiposity.

As shown by many authors, leptin can exert its action not only through ObR, but also through many other signaling systems [5,6]. In the context of the most aggressive breast

cancer, it is important that leptin could crosstalk with HER2 either through ObR, HER1, and JAK2 [22,29].

HER2 is a major marker of aggressive breast cancer and an important pharmaceutical target [28,46]. HER2-targeted therapies with trastuzumab improved survival of patients with HER2 overexpressing metastatic breast cancer and early-stage breast cancer. However, primary or treatment-induced resistance to this drug often occurs [28,47]. The mechanisms of this resistance seem to include increased activation of other signaling systems, for instance, overexpression of IGF-1 receptors, increased synthesis of EGF or TGF-alpha, mutation of PTEN phosphatase resulting in constitutive PI-3K activation [28,47]. Thus, targeting alternative signaling systems in HER2-positive tumors might prove beneficial; indeed, clinical trials exploring such options are underway [28,48].

However, the interaction between HER2 and the leptin system has not been well explored in breast cancer. Here we report that in breast cancer cell lines, endogenous coexpression of HER2 and ObR is common, but cells expressing very high levels of HER2 appear to express low levels of ObR. In MCF-7 cells, which contain relatively high levels of ObR [32] and moderate levels of HER2, high physiological doses of leptin can induce HER2 tyrosine phosphorylation. Similar transactivation was recently described in SK-BR-3 cells [29]. In the case of MCF-7 cells, we show that leptin-dependent stimulation of HER2

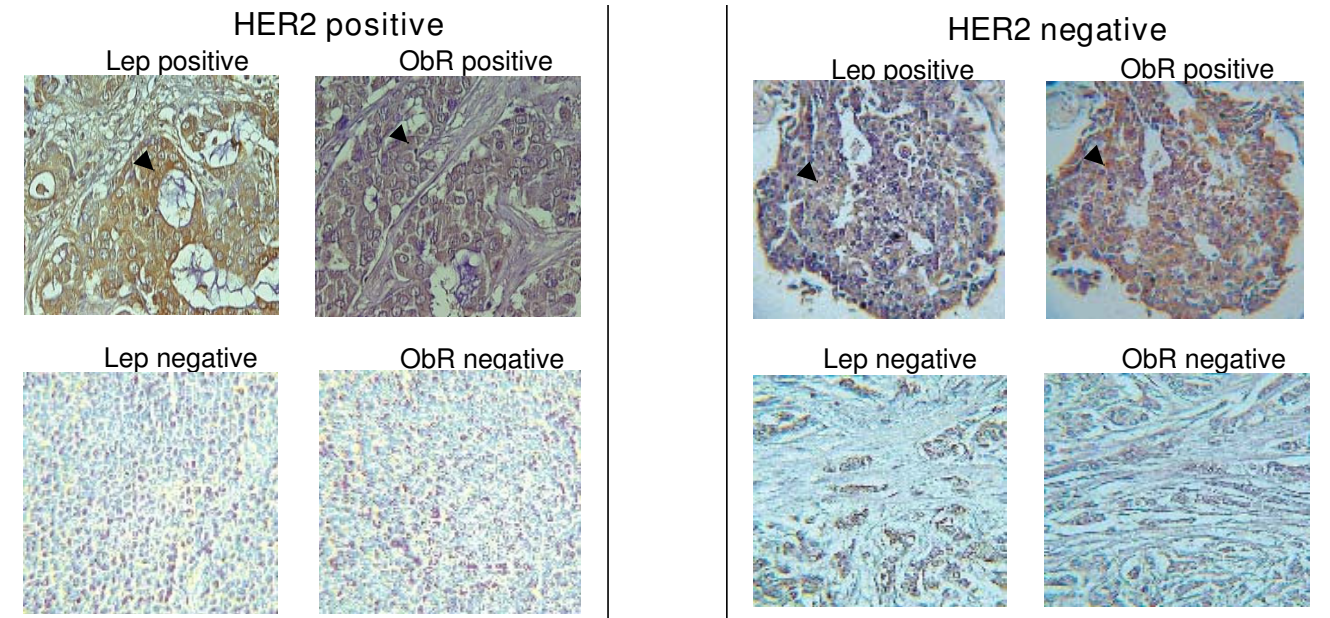


Figure 4
Expression of the leptin/ObR system in HER2-positive and -negative breast cancer. The expression of leptin and ObR was studied by IHC, as described in Materials and Methods in HER2-positive and HER-2-negative samples. The magnification level is 40x. Arrows indicate the example areas of leptin and ObR staining.

Table 3: Associations between leptin, ObR, leptin/ObR and different clinicopathological parameters

		Leptin		ObR		
		N	Positive (%)	p-value	Positive (%)	p-value
Diameter (mm)				0.061		0.137
	≤ 10 mm	9	55.6		66.7	
	> 10 mm	42	85.7		88.1	
N				> 0.5		0.242
	N0	19	84.2		94.7	
	N+ (N > 0)	33	78.8		81.8	
LVI				> 0.5		0.473
	Negative	24	79.2		87.5	
	Positive	27	77.8		77.8	
Menopausal status				> 0.5		> 0.5
	Postmenopausal	15	80.0		86.7	
	Premenopausal	39	82.1		84.6	
Histotype				> 0.5		0.432
	Other	3	66.7		66.7	
	Ductal (invasive)	45	80.0		84.4	
	Inflammatory	4	100.0		100.0	
	Intraductal	3	66.7		66.7	
	Lobular (invasive)	4	75.0		75.0	
G				0.495		0.366
	G1	7	85.7		100.0	
	G2	18	88.9		88.9	
	G3	26	73.1		76.9	
Ki-67				> 0.5		> 0.5
	0–15%	33	75.8		81.8	
	16–25%	9	88.9		88.9	
	26–100%	14	85.7		85.7	
ER/PgR				> 0.5		> 0.5
	ER-/PgR-	12	75.0		83.3	
	ER-/PgR+	1	100.0		100.0	
	ER+/PgR-	6	83.3		83.3	
	ER+/PgR+	37	81.1		83.8	
T				0.019		0.400
	pT1	20	70.0		80.0	
	pT2	23	91.3		91.3	
	pT3	2	0.0		50.0	
	pT4	8	100.0		87.5	
	pTx	1	100.0		100.0	
	TIS	3	66.7		66.7	
HER2				0.342		0.306
	Negative	28	85.7		89.3	
	Positive	31	74.2		77.4	

For each level of each clinicopathological variable, the total number (N) of patients is given together with the percentage of subjects positive for leptin or ObR expression. Abbreviations as in Tab. 2.

could be facilitated by proximity or direct interaction of HER2 and ObR, as demonstrated by colocalization and coimmunoprecipitation experiments. The exact mechanism of HER2 phosphorylation by liganded ObR is not known, but one could speculate that intermediate cellular tyrosine kinases, such as JAK2 (normally binding to activated ObR) or src (possibly interacting with either ObR1 or ObRs), could be involved. On the other hand, in cells expressing low ObR1 levels, e.g., SK-BR-3 cells, leptin

appears to transactivate HER2 via HER1 and JAK2 pathways [29].

In this study, we evaluated whether HER2/ObR crosstalk observed in cellular models could occur in human breast cancer in vivo. We noted that the leptin/ObR system is coexpressed with HER2 in a large fraction of breast cancers, which supports the possibility of intratumoral ObR/HER2 interactions. Notably, the presence of leptin/ObR

was numerically more frequent in larger tumors. However, perhaps due to the relative small sample of tumors analyzed, we were unable to detect any associations between leptin/ObR and either tumor grade, ER α /PgR, or metastasis, reported by different authors previously [18-20].

The statistical analysis employed deserves some comments. The Fisher's exact test is the dominant method for making inferences from 2×2 tables where the number of observations is small. The test assumes that both of the margins in a 2×2 table are fixed by construction ("conditional" test), but if an alternative process generates the data, the test might not provide a correct coverage. Nonetheless, the Fisher's exact test is often used, since in general, it generates a conservative result. In our case no margins of the 2×2 tables were fixed, so that an "unconditional" exact test would be more appropriate. To validate our analysis, we performed such test, which gave, as expected, a more significant result, in particular when analyzing the association between leptin and ObR in HER2-negative tumors ($p = 0.024$ vs. $p = 0.045$ obtained using the Fisher's exact test).

The finding that both leptin and ObR can be found not only in HER2-positive but also in HER2-negative tumors suggests that leptin/ObR and HER2 systems are controlled by separate mechanisms. Interestingly, in our latest screening of ~90 "triple-negative" tumors (lacking ER α , PgR, and HER2 expression), we detected leptin and ObR in most cases analyzed (manuscript in preparation, data not shown). In this cellular context where hormonal and traditional targeted therapy are not applicable, the leptin system might constitute a new attractive target.

Conclusion

In summary, our results suggest the existence of crosstalk between HER2 and the leptin system in breast cancer. This notion implicates targeted anti-ObR therapies as possible future therapeutic options, especially in tumors that become resistant to targeted HER2 therapy. Such therapeutic approaches could be especially effective in patients expressing high physiological levels of leptin (100–300 ng/mL), characteristic for the overweight and obese phenotype.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

EF and AM carried out molecular and IHC studies, contributed to manuscript writing and editing; MT, AA, VP, and B DS carried out IF and leptin/ObR IHC studies; AR and MF B prepared pathology samples and evaluated IHC for different markers; RM performed statistical analysis of

all data; AM M, GL C and AG participated in the design of the study and edited the manuscript; ES conceived and coordinated the study and drafted the manuscript.

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